Gonadal Restructuring During Sex Transition in California Sheephead: a Reclassification Three Decades After Initial Studies

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Abstract.—California Sheephead, *Semicossyphus pulcher*, is a monandric proto-gynous hermaphrodite and a commercially and recreationally valuable labrid. Gonadal functionality of Sheephead through sex change was reclassified into nine classes using current criteria for categorization. Female ovaries were classified as immature, early maturing, mature, and regressing/recovering classes. Transition from female to male and subsequent male development was divided into early, mid and late transitional, developing/active male and regressing/recovering male. Reproductive states in Sheephead were correlated with estradiol (E2) and 11-keto testosterone (11-KT) concentrations in the blood plasma. All sexes had low E2 concentrations in the fall /winter seasons; in transitional and male individuals, levels remained low throughout the year. In contrast, female E2 concentrations were elevated in spring and peaked in the summer. Concentrations of 11-KT were variable throughout the year; however, females had significantly lower levels in the summer. This study allows a better understanding of the current state of California Sheephead in a heavily fished area. Knowledge of a species' reproductive characteristics is important in evaluating the sustainability of a population as it can set a baseline for reproductive potential. This research takes a critical step in gathering and organizing reproductive data such that it may be used in future studies for comparing reproductive potential across the range of the California sheephead.

Introduction

Over the last 50 years, research efforts have focused on the process of post-maturational sex changes in fishes. This process has been described by histological studies of the gonads, as well as through characterization of body morphology (Moe 1969; Choat and Robertson 1975; Warner 1975; Shapiro 1981, 1987; Ross 1987; Hastings 1989; Sadovy and Colin 1995; Bruslé-Sicard and Fourcault 1997; Liu and Sadovy 2004; Mackie 2006). The mechanism and direction by which different fishes change sex varies significantly (Munday et al. 2006), making a descriptive analysis of sex change characteristics and methods for quantifying sex change both fundamental and foundational for further research of any particular hermaphroditic species.

California Sheephead, *Semicossyphus pulcher* (Ayres), is a commercially and recreationally valuable labrid found from Monterey Bay, California, to Cabo San Lucas, Mexico (Miller and Lea 1972). Sheephead are an epibenthic species that generally utilize sand-rock reef habitats for foraging while taking shelter and reproducing in and over rocky reefs habitat (Topping et al. 2005). Because adult *S. pulcher* feed heavily on

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urchins, they are an important species for indirectly regulating kelp growth in southern California's coastal waters (Cowen 1983, Dayton et al. 1998). Overfishing has resulted in depletion of many populations of Sheephead throughout southern California (Alonzo et al. 2004; Hamilton et al. 2007).

As monandric, protogynous hermaphrodites, California Sheephead can transition from a reproductively functional female to a functional male during the course of a lifespan in response to social factors (Warner 1975; Adreani et al. 2004). The transitional phase has been reported in other sex-changing teleosts to take from 2–3 weeks to several months (Robertson 1972; Mackie 2003; Sadovy and Shapiro 1987; McBride and Johnson 2006). While sex hormones have not been previously examined during transition in Sheephead, we expect that changes in steroid hormone concentrations; specifically, 17β-estradiol (E2) and 11-ketotestosterone (11-KT) are related to sex change due to the total degradation of the ovaries and the appearance of testes.

Although ambisexuality is widely displayed in teleost fishes and there have been numerous studies describing the sex change process in fishes, comparisons within and among species have been limited due to inconsistencies in gonad class description and terminology. Recently, proposals for a classification system of gonad development in both gonochoristic and hermaphroditic fishes have been made (e.g., Brown-Peterson 2006; Barbieri et al. 2006; Brulé and Colás-Marrufo 2006). To reflect the growing body of scientific knowledge, and to provide consistency among studies, it is important that attempts be made to bring descriptions of fish sexual development in line with more current terminology.

Reproduction in California Sheephead was first described more than 30 years ago using histological methods, and as individuals change from female to transitional to male, several classes of development were identified (Warner 1975). This study re-examines Sheephead gonadal development in light of several decades of environmental and anthropogenic pressures, with the goal to update Sheephead gonadal development descriptions to aid in future studies of the sex-change process within this species. In addition, we sought to correlate changes in the gonads with alterations in plasma concentrations of sex steroid hormones. Strong correlations between sex hormones and gonad state could provide a non-lethal means to determine reproductive state and this may be a useful tool for both future researchers and fishery managers.

Due to their economic significance and population decline, a comprehensive and current model of Sheephead reproductive function at the gonadal level is critical for proper future management and research of the species. Such a model could be valuable in determining the health of the Sheephead population, in terms of reproductive capacity. In this paper, we identify and reclassify the reproductive classes of Sheephead caught near Santa Catalina Island, California, using current terminology. In doing so, we take the first steps in determining the current reproductive profile of the Santa Catalina Island Sheephead population.

Materials and Methods

Animals

California Sheephead were obtained within a 2 km radius of Bird Rock, Santa Catalina Island, California (33°29′N, 118°27′W) from October 2004 through October 2005. Fish were caught by hook and line at depths ranging from 5 to 40 m, or by baited traps at depths of 14 to 21 m. Once caught, fish were brought to the surface and their swim bladders were vented with a hypodermic needle to relieve the effects of barotrauma.

Sheephead were then transported in a 60 L cooler to the laboratory for dissection. The maximum time between capture and dissection was 5 hours. Each fish was euthanized in an ice-slurry immediately prior to dissection.

Each fish was immediately assigned to one of three gender groups (female, transitional, male) based on previously described external morphological characteristics and coloration (Warner 1975). In addition, blood was collected via the caudal vein prior to euthanization to assess the seasonal relationship of certain steroid hormones (E2 and 11-KT) to gonad function. Blood samples were centrifuged at 1500 rotations per minute for 5 minutes and blood plasma was removed. Plasma samples were stored at $-80°C$ until collections were complete and hormone assays could be conducted.

Tissue Processing

After euthanization, bilobate gonads were excised, cleaned of connective tissue and weighed. Portions from each end and the midsection of the gonad lobes were immersed in 10% formalin to maintain protein structure and composition. Gonads were then processed for histological analysis.

After 10 days in formalin, gonads were washed in phosphate-buffered saline solution (PBS), dehydrated in a graded series of ethanol solutions, and embedded into paraffin. Gonads from each individual were cut in 6 µm thick sections and mounted onto slides. Sections from each gonad were mounted serially with 60 µm of tissue between each mounted section. All sections were mounted onto Superfrost-plus microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were placed through a series of xylene and ethanol washes in order to hydrate the tissues, and stained using hematoxylin and eosin.

Gondal Tissue Characterization

Female, male and transitional gonadal tissues were identified and characterized based on previous work (Warner 1975; Guraya 1986; Sadovy and Shapiro 1987; Nakamura et al. 1989; Taylor et al. 1998), and recent efforts to standardize terminology in describing gonad tissue development were consulted (e.g., Brown-Peterson 2006; Barbieri et al. 2006; Brulé and Colás-Marrufo 2006). The terms "class" and "stage" were used to define the development of the gonad and the gametes, respectively.

Follicle Counts and Characterization of Transitionals/Males

Serial sections of female gonads were used to analyze the relative density of ovarian structures. Class of gonadal development was recorded using brightfield microscopy, and the number of structures for each follicle stage within or overlapped by a 1 mm^2 guide was counted. Three arbitrarily selected locations within each tissue section were counted. For each ovary, the average relative density of each ovarian structure per section was determined from the six representative gonadal cross sections counted per individual.

Transitional individuals and males were assessed by observing both the level of gonadal reconstruction (degeneration of ovarian structures and generation of testicular tissue) and the stage of spermatogenesis. Determination of sex was made based on the most predominate stage of spermatogenesis observed in the six representative sections of gonad for each animal.

Hormone Analysis

Estradiol concentrations in the blood plasma were measured using DSL-4800 Ultra-Sensitive Estradiol RIA ^{125}I double antibody kits (Diagnostic Systems Laboratories, Inc.,

Webster, TX). Samples were assayed in duplicate and radioactivity was measured using a Perkin-Cobra II gamma counter (Packard Instruments Co., Boston, MA). SigmaPlot 8.0 software (SPSS Inc., Chicago, IL) was used to generate a standard curve in the four-parameter logistic curve function and determine hormone concentrations. Assay standards and controls were within the normal limits (Moffatt-Blue et al. 2006; Schmidt and Kelley 2001), with a 2.2 pg/mL lower limit of detection and low (0.64–2.40%) cross-reactions to other steroids.

Plasma 11-KT concentrations were measured using competitive enzyme immunoassays kits (ACE^{TM} kits from Cayman Chemical Co., Ann Arbor, MI). Each sample was assayed in duplicate and two dilutions of each sample with between 20 and 80% B/B0 values were averaged. Plates were read using a Powerwave XS Bio-Tek microplate spectrophotometer at 412 nm. Raw data (absorbances) were exported to Microsoft Excel spreadsheets and analyzed using 2006 Cayman Chemical Enzyme Immunoassay (EIA) Tools software, (Cayman Chemical Co.). The average intra-assay variability was 8.3% and the lower limit of detection was 5.3 pg/mL.

Data Analysis

For most analyses, fish were grouped according to the season in which they were caught; *fall* (October through December), *winter* (January through March), *spring* (April through June), and *summer* (July through September). Changes in seasonal follicle densities were analyzed using ANOVA analysis. A Student-Newman-Keuls post-hoc test was used to compare differences in follicle densities between seasons ($\alpha = 0.05$). Seasonal E2 concentrations were analyzed using a Friedman's two-way non-parametric ANOVA ($\alpha = 0.05$) to compare effects of season and gender. Kruskal-Wallis tests were used post-hoc to compare differences in E2 levels among sexes by season, and a Mann-Whitney test was used to compare differences among females across seasons. Concentrations of 11-KT for females, transitional individuals and males were square-root transformed and a two-way ANOVA ($\alpha = 0.05$) was used to assess differences.

Results

Catch Assessment

A total of 133 California Sheephead were caught over the course of a year at Santa Catalina Island, of which 94 adult fish were used in the histological analysis. Based on observations from these mature individuals, as well as several immature gonads, the development from female to male was characterized on a continuous scale ranging over nine gonad development classes (Table 1).

Ovarian Morphology and Seasonal Changes

Female gonad development comprised the first four of the nine classes, and determination of gonad class was made using the predominant oocyte structural stages present in the ovary (Figure 1). Female ovaries were classified as *immature* (Figure 1A), *early maturing* (Figure 1B), *mature* (Figure 1C), and *regressing/recovering* (Figure 1D) classes. The *regressing/recovering* ovaries of females not transitioning to males return to the *early maturing* class. Oocytes move through four stages of development before release during spawning (*chromatin nucleolar oocytes*, *perinucleolar oocytes*, *yolk vesicle oocytes*, *yolk globular oocytes*), whereas follicles with unreleased oocytes underwent atresia (Figure 1D) within the ovary. Post-ovulatory follicles were also noted in gonads found in the mature ovary class (Figure 1C).

Table 1. Reproductive classification of the protogynous hermaphrodite, California Sheephead (*S. pulcher*). Reproductive class is based on gonad morphology and histological examination of gonad structures.

| Sex	Reproductive class	Gonad characteristics
Female	1. Immature	Gonads contain primary growth stage oocytes only, including chromatin nucleolar oocytes (CO) and perinucleolar oocytes (PO). Ovarian walls are thin and lamellae are tightly packed. Cytoplasm is generally basophilic and stains darker than more mature cytoplasm.
	2. Early maturing	Yolk vesicle (cortical alveolar) oocytes (YVO) are notable, along with CO and PO. As follicles begin maturing, they become less basophilic and appear slightly lighter in coloration to surrounding primary growth oocytes.
	3. Mature	Yolk globular oocytes (YGO) are present along with all other follicular development stages. Post-ovulatory follicles (POF) are also present. Follicular cytoplasm is relatively acidophilic and stains much lighter than previous stages.
	4. Regressing/recovering	Many CO and PO are present, along with widespread atresia of residual YVO and YGO.
Transitional	5. Early transitional	A high level of atresia and a high disruption of tissues. Some CO and PO are present. Few to no spermatocysts notable. Few specimens were noted in this stage.
	6. Mid transitional	Some CO and PO still present, along with many atretic follicles. Several spermatocysts (SC) of earlier stages in spermiogenesis are notable in the lamellae, however few spermatids and maturing sperm.
	7. Late transitional	CO and PO no longer present, however pockets of large atretic follicles are still notable. SC have become more numerous and may contain all stages of spermatogenesis. The tissue is primarily testis.
Male	8. Developing/Active	SC during developmental stages become dense and numerous, with all stages of spermatogenesis potentially present. As development continues males become reproductively active; spermatids (ST) and spermatozoa (SZ) become more prevalent. Few to no atretic follicles remain in the gonads.
	9. Regressing/recovering	Residual spermatocysts (RSC) remain containing spermatozoa. Primary and secondary spermatocytes (1SY and 2SY) are most prevalent spermatogenic stage, and some SC containing 1SY can also be observed.
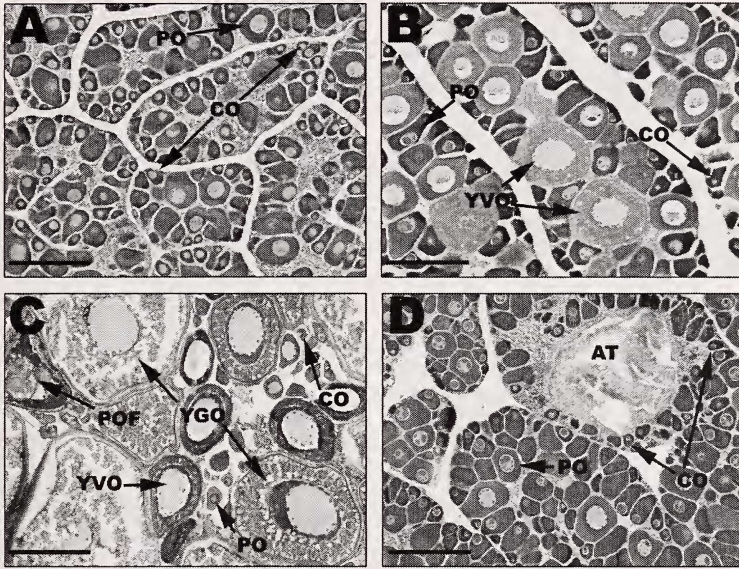
Fig. 1. Representative photographs of female California Sheephead ovarian follicle development. Bars in the lower left corner of each photo represent 200 micrometers. (A) Immature female gonads. (B) Maturing ovaries. (C) Mature ovaries. (D) Regressing/recovering ovaries. AT = atretic follicle. CO = chromatin nucleolar oocyte. PO = perinucleolar occyte. POF = post-ovulatory follicle. YVO = yolk vesicle oocyte. YGO = yolk granule oocyte.

Histological analysis of gonads throughout the year showed seasonal changes within the structure of female gonads (Figure 1). During the winter season, adult females exhibited only primary growth oocytes (chromatin nucleolar oocytes and perinucleolar oocytes) and atretic follicles (Figure 1D). Follicle development (ovarian follicle stage densities – yolk globular oocytes and atretic follicles) differed across seasons (Kruskal-Wallis: $H = 17.71$, d.f. = 3, $p = 0.001$; Figure 2A; Kruskal-Wallis: $H = 10.50$, d.f. = 3, $p = 0.015$; Figure 2B). Atretic follicle count data were normalized by adding 0.1 to all values and log transforming. Oocyte development became evident during the spring and summer months and peaked in the summer, with relative mean density of summer yolk globular oocytes 8.1-fold greater than in spring (Figure 2A). No yolk globular oocytes were observed in winter samples. The densities of atretic follicles peaked in the fall and winter and were lowest in the summer (Figure 2B).

Morphological Development and Seasonal Changes of Testes

Gonadal transition from female to male and subsequent male development was divided into five classes, with the first three classes considered transitional. During the early stages of sexual transition, chromatin nucleolar oocytes, perinucleolar oocytes, and atretic follicles were observed within the tissue (Figure 3A), along with developing spermatocysts that became more common in mid-transitional fish (Figure 3B). In early transitional individuals, the gonads were greatly reduced in size. In late transitional individuals, testicular tissue was more prevalent and later stages of spermatogenesis were noted in some specimens, including primary and secondary spermatagonia, primary and secondary spermatocytes, spermatids and occasionally spermatozoa (Figure 3C). The presence of these cell types indicate that fish in late transition may be reproductively
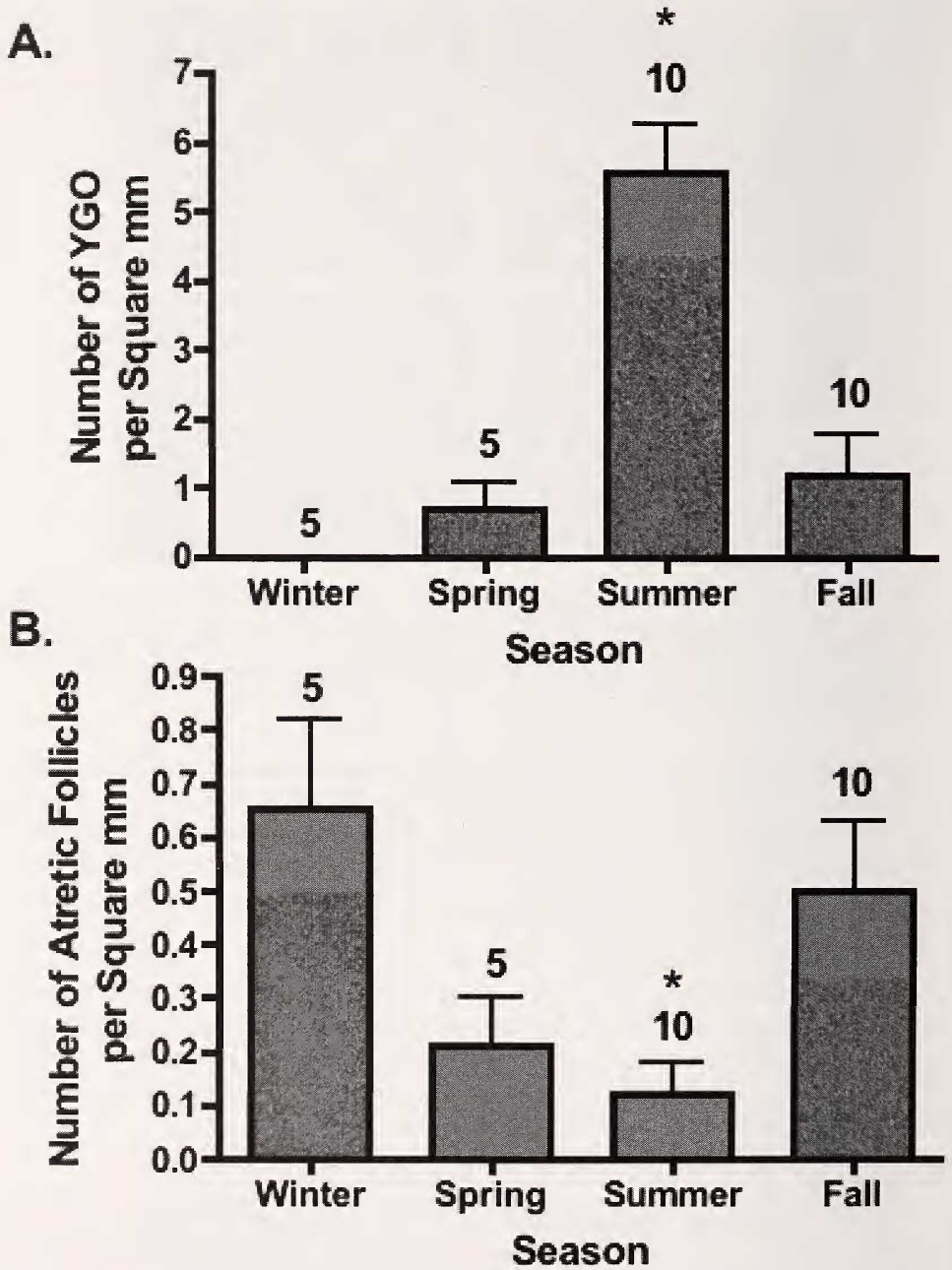
A.

B.

Fig. 2.   (A) Mean density of yolk-globular oocytes (cells per mm^2) for mature females over seasons; (*) represents significant difference from all other groups. (**B**) Mean density of atretic follicles (cells per mm^2) for mature females over seasons; (*) represents significant difference from winter and fall. Error bars represent one Standard Error. Numbers above each bar represent the sample size.
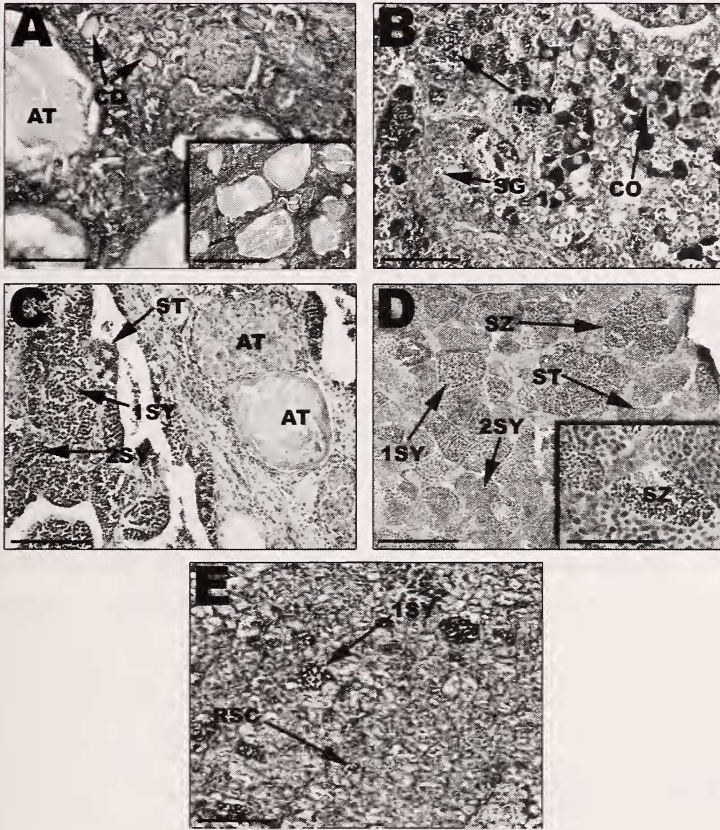
Fig. 3. Representative photographs of male California Sheephead testicular development. Bars in the lower left corner of each photo represent 100 micrometers. Inset bars in (A) and (D) represent 400 micrometers and 50 micrometers, respectively. (A) During the earliest noted transition class very high levels of atretic follicles are noted, few early stage oocytes, and massive tissue reorganization. (B) As the transition progresses, spermatogonia and primary spermatocytes can be observed among early stage oocytes. (C) In late transition, early stage oocytes are very rare, with further stages of spermatogenesis noted. (D) Active males commonly exhibit the final stages of spermatogenesis. (E) Inactive males contain mostly residual spermatocytes and early stage spermatogenic tissue. AT = atretic follicle. CO = chromatin nucleolar oocyte. RSG = residual spermatocyte. SG = spermatagonia. ST = spermatid. 1SY = primary spermatocyte. 2SY = secondary spermatocyte. SZ = spermatozoa.

functional as male. In addition, primary stage oocytes were no longer found in the gonads of fish classified as late transitional, although oocyte atresia was still apparent (Figure 3C). Gonads of fish classified as mature males showed no residual atretic follicles (Figure 3D). During the summer breeding months, the male gonad was characterized by dense spermatocysts of all developmental stages (e.g., Figure 3D); while during the winter non-breeding months the mature male testis contained mainly spermatogonia, residual spermatozoa, and some primary spermatocytes (Figure 3E).

Hormone Concentrations

No measurable levels of E2 were detected in most transitional individuals or in males. Concentrations of E2 differed significantly by season ($F_{3,90} = 7.62$, $p = 0.0001$) and by gender ($F_{2,90} = p < 0.0001$; Figure 4A). There was no difference in E2 concentrations
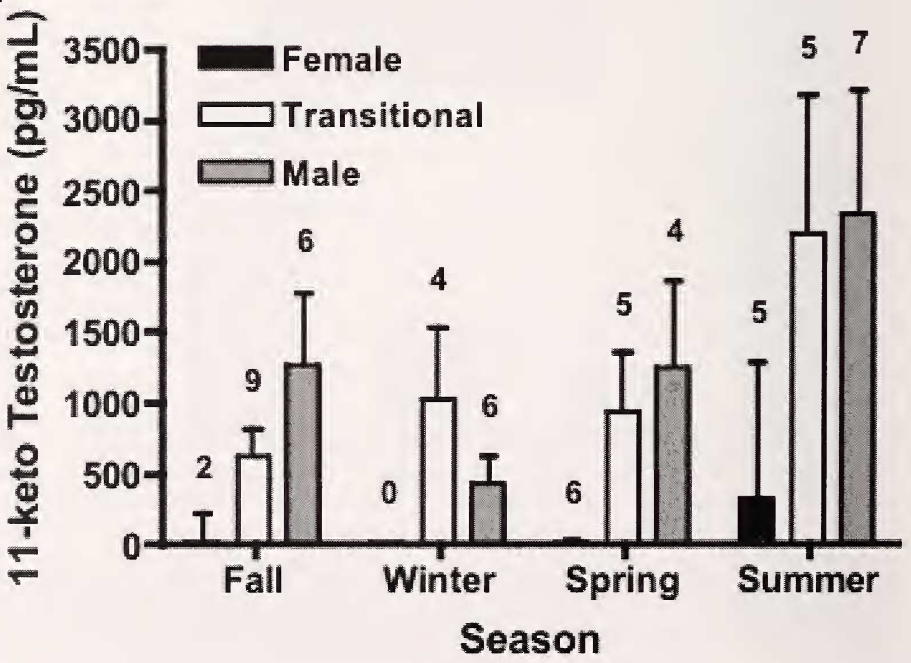
A.
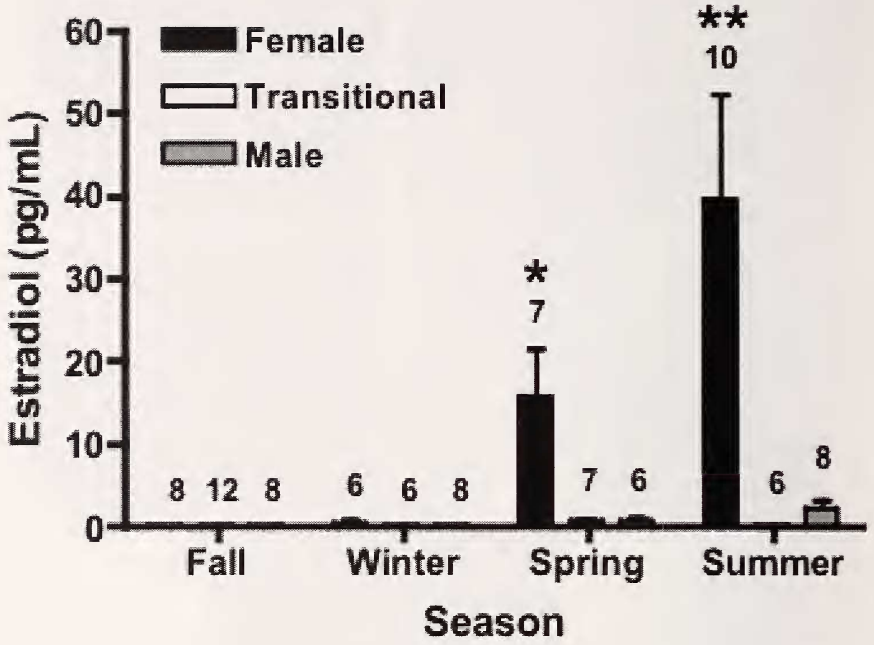
B.

Fig. 4. Mean seasonal plasma E2 (A) and 11-KT (B) concentrations of female, transitional and male Sheephead at Santa Catalina Island. Error bars represent one Standard Error and (*) represents significant difference. Numbers above each bar represent the sample size.

among genders in fall (Kruskal-Wallis: $H = 0.00$, d.f. $= 2$, $p = 1.0$) and winter seasons (Kruskal-Wallis: $H = 2.33$, d.f. $= 2$, $p = 0.31$); however, females had significantly greater E2 levels than transitional individuals and males in spring (Kruskal-Wallis: $H = 9.7$, d.f. $= 2$, $p = 0.008$) and in summer (Kruskal-Wallis: $H = 15.76$, d.f. $= 2$, $p = 0.000$). Female E2 concentrations peaked in the summer and levels were significantly greater in the summer than in the spring (Mann-Whitney: $W = 42.5$, $p = 0.05$).

There were significant differences in 11-KT by gender ($F_{2,60} = 17.5$, $p < 0.001$) and season ($F_{3,60} = 0.033$, $p = 0.033$; Figure 4B). Concentrations of 11-KT were significantly higher in males (Tukey: $p < 0.001$) and transitionals (Tukey: $p < 0.001$) than females overall; however, only during the summer season (Tukey: $p = 0.049$).

Discussion

Populations of California Sheephead have been significantly reduced due to overfishing and this decline has resulted in a reduction in the size at transition, an altered sex ratio, and likely an increase in the rate of transition (Hamilton et al. 2007). The current data present an updated assessment of California Sheephead protogynous sexual development within the context of these recently reported changes in population structure and are intended for use in future studies of California Sheephead populations and other temperate teleost species. We have established and updated the definition of female, transitional, and male gonad classes concomitant with the current and revised gonadal terminology. Critically, these classifications reveal changes that have occurred in the Santa Catalina Island population that mirror recently reported alterations in population structure at other heavily fished islands (Hamilton et al. 2007). Although we cannot determine from this study the time required to change sex or the impact on the overall reproductive capacity, our findings indicate that there is a period of reproductive inactivity involved during gonadal remodeling. Future studies should examine the effects of changing sex related to the timing of sex change on the reproductive capacity of Sheephead at the individual and population level.

A recent consortium for uniformity and consistency in the classification of teleost gonad structure has sought to reduce the discrepancies present across histological studies of different fishes (e.g., Nunez and Duponchelle 2009; Brown-Peterson 2006; Barbieri et al. 2006; Brulé and Colás-Marrufo 2006). While the refined terminology was originally predicated upon non-hermaphroditic species, we have applied the current terminology to the sexual development and transition of California Sheephead gonads. The current study categorized sex change into nine classes, as opposed to the prior eight (Warner 1975). The sample sizes of transitional individuals in the present study exceed what was observed previously, allowing the classification of two more transitional classes, while deleting one male (post-spawning) class as it was never observed during this study and is thought to be a relatively short-lived phase.

Our histological data suggests that sex change in Sheephead is unidirectional. While the exact timing of transition from functional female to functional male remains unclear, ovarian remnants in later stage transitionals are atretic as compared to ovarian structures with further developmental potential (chromatin nucleolar oocytes, perinucleolar oocytes, and mature oocytes) found in males of other fish taxa known to exhibit bi-directional sex change (Cole 2003; Wittenrich and Munday 2005). It is possible that in the later stages of the transition, fish are functionally male but maintain an intersexual gonadal appearance (Sadovy de Mitcheson and Liu, 2008). Among late transitional fish, the presence of later stages of spermatogenesis along with ovarian remnants is an

important criterion for determining fish still within the transitional classes (Sadovy and Shapiro 1987). Behavioral and further histological studies must be coordinated to determine if mature sperm are released during mating among Sheephead that exhibit these gonadal characteristics. Among early and mid- transitional fish, where ovarian tissue persists and still includes oocytes in early developmental stages and spermatogenic tissue remains sparse, the potential to function as a male remains unknown but is likely far lower than those in the late transitional stage. Categorizing transition from female to male into three classes as opposed to leaving it as one (Warner 1975) allows for better future estimates of the reproductively non-active percentage of a Sheephead population, especially for those in transition during the breeding season.

Concentrations of E2 were elevated in spring and peaked among females in the summer as compared to the non-breeding season months; levels were not significantly different from males or transitionals in the fall and winter months (Figure 4). This seasonal peak corresponds with maximal ovarian function as observed in mature gonads from summer fish. However, it appears that females begin to prepare for the breeding season in late spring because ovarian function (i.e. E2 concentrations and yolk globular oocytes) is higher in spring than in fall and winter (Figure 1). During spring and summer, concentrations of E2 were significantly higher in females as compared to both males and transitionals; for transitionals this held true regardless of transition class. This suggests that follicular function, as measured by E2 production, declines rapidly as females undergo transition. Indeed, atretic follicles were observed in all classes of transitioning fish: early, mid, and late. Interestingly, the decline in plasma E2 among transitional fish as compared to females was observed during the breeding as well as the non-breeding season. Together, these data suggest that once an individual initiates sex change, there is a rapid and lasting decline in female gonadal function.

While 11-KT appeared to peak in the summer breeding season as opposed to non-breeding months these increases were not significant (Figure 4). Typically, 11-KT is the primary androgen in male teleosts, and generally is highest concomitant with the breeding season to facilitate reproductive behaviors and physiology (Borg 1994). In California Sheephead, however, 11-KT concentrations were widely variable, without a clear summer peak in males. It may be that the pulsatile nature of this hormone resulted in the high variability observed across males; however, the lack of a significant peak during the breeding season may also suggest that other androgens are playing a larger role in reproduction in this species.

In summary, these data show for the first time that at the heavily-fished Santa Catalina Island Sheephead population, gonadal function corresponds to peak hormone function, particularly in females. In light of the substantial changes in population structure at Santa Catalina Island as recently reported for Sheephead (Hamilton et al. 2007), we have reexamined the classification of gonadal morphology and updated terminology. Our findings will allow for better comparisons of reproductive capacity across Sheephead populations. Our endocrine data suggest that hormone analysis may provide a non-lethal method of wide-scale sampling for Sheephead. Together, these methods may be used across the Sheepheads range to determine variations is social structures of different populations and possibly better understand the effects of sex biased fishing pressure on these populations.

Acknowledgments

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